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Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/s00253-005-0233-1

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2006

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Gutierrez, J., Larsen, R., Cintas, L. M., Kok, J., & Hernandez, P. E. (2006). High-level heterologous production and functional expression of the sec-dependent enterocin P from Enterococcus faecium P13 in Lactococcus lactis. Applied Microbiology and Biotechnology, 72(1), 41-51. https://doi.org/10.1007/s00253-005-0233-1

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Appl Microbiol Biotechnol (2006) 72: 41–51
DOI 10.1007/s00253-005-0233-1

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High-level heterologous production and functional expression of the *sec*-dependent enterocin P from *Enterococcus faecium* P13 in *Lactococcus lactis*

Received: 9 September 2005 / Revised: 20 October 2005 / Accepted: 22 October 2005 / Published online: 17 January 2006
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Abstract Enterocin P (EntP), a *sec*-dependent bacteriocin from *Enterococcus faecium* P13, was produced by *Lactococcus lactis*. The EntP structural gene (*entP*) with or without the EntP immunity gene (*entiP*) was cloned in (1), plasmid pMG36c under control of the lactococcal constitutive promoter P_{32}, (2) in plasmid pNG8048e under control of the inducible P*nisA* promoter, and (3) in the integration vector pINT29. Introduction of the recombinant vectors in *L. lactis* resulted in production of biologically active EntP in the supernatants of *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* NZ9000, and the coproduction of nisin A and EntP in *L. lactis* subsp. *lactis* DPC5598. The level of production of EntP, detected and quantified by specific anti-EntP antibodies and a non-competitive indirect enzyme-linked immunosorbent assay, by the recombinant *L. lactis* strains depended on the host strain, the expression vector, and the presence of the *entiP* gene in the constructs of the recombinant *L. lactis* strains. The highest amount of EntP was produced with derivatives containing *entP* and *entiP*, for both *L. lactis* IL1403 and *L. lactis* NZ9000. These derivatives produced up to five- to six-fold more EntP than *E. faecium* P13. Mass spectrometry analysis revealed that EntP purified from *L. lactis* IL1403 (pJP214) has a molecular mass identical to that purified from *E. faecium* P13, suggesting that the synthesis, processing, and secretion of EntP progresses efficiently in recombinant *L. lactis* hosts.

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Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides secreted by bacteria, and because bacteriocins produced by lactic acid bacteria (LAB) inhibit not only closely related species but also Gram-positive spoilage bacteria and food-borne pathogens, they attract considerable interest for their potential use as natural and nontoxic food preservatives (Cleveland et al. 2001; O'Sullivan et al. 2002). LAB bacteriocins or bacteriocin-producing strains, either alone or in combination with other antimicrobial barriers, may be useful tools for food preservation. The heterologous production of bacteriocins in different hosts could lead to: (1) increased bacteriocin production, (2) production of bacteriocins in safer hosts, (3) construction of multibacteriocinogenic strains with a wider antagonistic spectrum, (4) better adaptation of the selected hosts to food environments, and (5) providing antagonistic properties for LAB already used as starter, protective, or probiotic cultures.

Most bacteriocins from LAB are synthesized as biologically inactive precursors or prepeptides containing an N-terminal extension. The mature peptides are often cationic, amphiphilic, membrane-permeabilizing molecules of 20 to 60 amino acid residues and are classified into three main groups: Class I consists of modified bacteriocins — the lantibiotics. Class II is comprised of nonmodified, heat-stable bacteriocins, which are divided into subclasses IIa (pediocin-like, strong antilisterial bacteriocins), IIb (two-peptide bacteriocins), and IIc (other peptide bacteriocins). Class III consists of the protein bacteriocins (Nes and Holo 2000; Cintas et al. 2001; Eijsink et al. 2002). N-terminal extensions of most lantibiotics and nonlantibiotics are of the so-called double-glycine type (leader sequence) and are cleaved off concomitantly with export across the cytoplasmatic membrane by dedicated adenosine triphosphate-binding cassette transporters (ABC-transporters) and their accesory proteins (Håvarstein et al. 1995; Venema et al. 1995). However, some class II bacteriocins, such as acidocin B (Leer et al. 1995), divergicin A (Worobo et al. 1995), bacteriocin 31

(Tomita et al. 1996), enterocin P (EntP) (Cintas et al. 1997), lactoccin 972 (Martínez et al. 1999), propionicin T1 (Faye et al. 2000), and enterolysin A (Nilsen et al. 2003), contain N-terminal extensions of the so-called *sec*-type (signal peptide), which are proteolytically cleaved concomitantly with bacteriocin externalization by the general secretory pathway or *sec*-dependent pathway (van Wely et al. 2001; Herranz and Driessen 2005). Several bacteriocins produced by enterococci, such as enterocins L50 (L50A and L50B), enterocin Q, and enterocin EJ97 (Cintas et al. 1998, 2000; Sánchez-Hidalgo et al. 2003), have been shown to be synthesized without N-terminal leader sequences, and may represent a new class of bacteriocins with a novel secretion mechanism.

Among the LAB, the enterococci produce a diverse and heterogeneous group of bacteriocins, coined enterocins, which are mutually different with respect to their antimicrobial activity, structure, processing, and secretion (Franz et al. 1999a; Cintas et al. 2001; Kawamoto et al. 2002; Nilsen et al. 2003). However, since enterocins may be produced by enterococcal species carrying antibiotic resistance genes and/or genes coding for potential virulence factors (Eaton and Gasson 2001; Franz et al. 2001; Shankar et al. 2002), interest in the heterologous production and functional expression of enterocins in other bacterial hosts is growing rapidly. In this context, the broad antimicrobial spectrum of EntP produced by *Enterococcus faecium* P13, a strain of food origin (Cintas et al. 1997), could find potential application in the food industry if the bacteriocin were produced by food-grade LAB. EntP is synthesized as a prepeptide consisting of a 27-amino-acid-signal peptide and the 44-amino-acid mature bacteriocin (Cintas et al. 1997), and has been known to dissipate the membrane potential of energized cells, and to form specific potassium ion-conducting pores in the cytoplasmatic membranes of target cells (Herranz et al. 2001a,b). In this paper, we report the cloning, production, and functional expression of EntP using different lactococcal expression vectors, and derivatives of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* as the production hosts.

Materials and methods

Bacterial strains, plasmids, growth conditions, and antimicrobial activity assays The LAB strains and plasmids used in this work are listed in Table 1. Lactococcal strains were grown in M17 medium (Oxoid) supplemented with 0.5% (wt/vol) glucose (GM17) at 30°C. *Enterococcus faecium* P13 was used as the source of EntP (Cintas et al. 1997), while *E. faecium* T136 (Casaus et al. 1997) was used as the indicator strain for determination of the EntP antimicrobial activity. Both microorganisms were propagated in MRS broth (Oxoid) at 32°C. Chloramphenicol or erythromycin (Sigma) was added to the cultures of *L. lactis* at 5 μg ml^{-1}. Antimicrobial activity of individual colonies was examined by the stab-on-agar test, as previously described by Cintas et al. (2000). Cell-free culture supernatants were obtained by centrifugation of cultures at 12,000×*g*, at 4°C for 10 min. They were adjusted to pH 6.2 with 1 M NaOH, filtered through 0.2-μm-pore-size filters (Whatman), and stored at −20°C until use. The antimicrobial activity of the supernatants was examined by an agar well diffusion test (ADT) and a microtitre plate assay (MPA), as previously described by Cintas et al. (2000), using *E. faecium* T136 (sensitive to EntP) and *L. lactis* MG1363 (resistant to EntP) as the indicator microorganisms. With the MPA, growth inhibition of the sensitive culture was measured spectrophotometrically at 620 nm with a microtitre Labsystems iEMS plate reader (Labsystems). One bacteriocin unit (BU) was defined as the reciprocal of the highest dilution of the bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin). Recombinant cultures of *L. lactis* NZ9000 were induced for production of EntP when they had reached an optical density at 600 nm (OD_{600}) of 0.5, using, as the inducer, either a 4×10^{-3}-fold diluted supernatant of *L. lactis* BB24 (NisA producer) or nisin Z purified to homogeneity from *L. lactis* NIZO 22186 (De Vos et al. 1993), both at 10 ng ml^{-1}.

Basic genetic techniques and enzymes Total genomic DNA from *E. faecium* P13 was isolated using the Wizard DNA Purification Kit (Promega). Plasmid DNA from the *L. lactis* strains was obtained using the High Pure Plasmids Isolation kit (Roche), as suggested by the manufacturer, but with the *L. lactis* cells previously suspended in solution A (Birnboim 1983) with lysozyme (10 mg ml^{-1}) and incubated at 55°C for 10 min, before following the kit instructions. All DNA-modifying enzymes were obtained from New England BioLabs or Roche and used as recommended by the supplier. Ligations were performed with the T4 DNA ligase (Roche). The *L. lactis* cells were transformed according to the method of Holo and Nes (1989), with a Gene Pulser and a Pulse Controller apparatus (Bio-Rad).

Recombinant plasmids derived from pMG36c The primers and inserts used for construction of the recombinant plasmids are listed in Table 2. Derivatives of plasmid pMG36c were constructed as follows: Primers LJ-1 and LJ-2 were used for polymerase chain reaction (PCR)-amplification from total genomic DNA of *E. faecium* P13 of a 261-bp *Sac*I–*Hin*dIII fragment (insert JP) containing the P_{32} ribosome binding site (RBS) and the EntP structural gene (*entP*) gene. Primers LJ-1 and LJ-3 were used for PCR-amplification from the same DNA target of a 533-bp *Sac*I–*Hin*dIII fragment (insert JPi), containing the P_{32} RBS and the *entP* and EntP immunity (*entiP*) genes. Fragments JP and JPi were digested with the indicated restriction enzymes and inserted in pMG36c cut with the same enzymes. The ligation mixtures were used to transform *L. lactis* IL1403. The proper clones, pJP26 and pJP214, respectively, were checked by bacteriogenicity tests, PCR, and sequencing of the inserts.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source and/or reference[b]
Strains		
E. faecium		
P13	Enterocin P producer	NBTA Cintas et al. (1997)
T136	Enterocins A and B producer; ADT and MPA indicator	NBTA Casaus et al. (1997)
L. lactis lactis		
IL1403	Plasmid-free derivative of IL594	Chopin et al. (1984)
DPC5598	Plasmid-free derivative of DPC4268	DPC Trotter et al. (2002)
BB24	Nisin A producer	NBTA Cintas (1995); Rodríguez et al. (1995)
L. lactis cremoris		
NZ9000	MG1363 pepN::*nisRK*	NIZO Kuipers et al. (1998)
MG1363	Plasmid-free derivative of NCDO712	IFR Gasson (1983)
LL108	MG1363 derivative carrying the pWV01 *repA* gene in its chromosome	Rug-MG Leenhouts et al. (1998)
JG1	Em^r, NZ9000 derivative carrying pINT29 in its chromosome	This work
JG5	Em^r, NZ9000 derivative carrying pJB3 in its chromosome	This work
JG8	Em^r, NZ9000 derivative carrying pJB19 in its chromosome	This work
Plasmids		
pMG36c	Cm^r, pMG36e derivative	Rug-MG Van de Guchte et al. (1989)
pJP26	Cm^r, pMG36c derivative carrying the PCR product JP (P_{32} ribosomal binding site and *entP* gene) under control of P32	This work
pJP214	Cm^r, pMG36c derivative carrying the PCR product JPi (P_{32} ribosomal binding site, *entP* and *entiP* genes) under control of P_{32}	This work
pNG8048e	Cm^r, Em^r, pNZ8048 derivative	NIZO Kuipers et al. (1998)
pJR22	Cm^r, pNG8048e derivative carrying the PCR product JR (*entP*) under control of P*nisA*	This work
pJR199	Cm^r, pNG8048e derivative carrying the PCR product JRi (*entP* and *entiP*) under control of P*nisA*	This work
pINT29	Em^r, ori^+, $repA^-$, partial sequence of *pepX* for integration	Rug-MG Leenhouts et al. (1998)
pJB3	Em^r, pINT29 derivative carrying the PCR product JB (P_{32} and *entP*) into *pepX*	This work
pJB19	Em^r, pINT29 derivative carrying the PCR product JBi (P_{32}, *entP* and *entiP*) into *pepX*	This work

[a] *ADT* agar well diffusion test, *MPA* microtitre plate assay, *Em* erythromycin, *Cm* chloramphenicol, *r* resistance, Ori^+ origin of replication of pWV01

[b] *NBTA* Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid (Madrid, Spain); *DPC* Teagasc Dairy Products Research Centre, Moorepark, Fermony, Co. (Cork, Ireland); *NIZO* Department of Biophysical Chemistry, NIZO Food Research (Ede, The Netherlands); *IFR* Institute of Food Research (Norwich, United Kingdom); *Rug-MG* Department of Molecular Genetics, University of Groningen (Haren, The Netherlands)

Recombinant plasmids derived from pNG8048e To construct the recombinant plasmids derived from pNG8048e, the purified pJP26 and pJP214 plasmids were used, respectively, as the PCR templates using the primer pairs RL-1 and RL-2, or RL-1 and RL-3, for generation of a 231-bp product containing the *entP* gene (insert JR), and for amplification of a 503-bp stretch of DNA containing the *entP* and *entiP* genes (insert JRi). The purified inserts were ligated as *Rca*I/*Bgl*I fragments into pNG8048e, previously digested with the enzymes *Nco*I and *Bgl*I, and the ligation mixtures were used to transform *L. lactis* NZ9000 electrocompetent cells. PCR with primers Nis-fw and Nis-rev confirmed the existence of the adequate pNG8048e-derivatives (pJR22 [containing JR] and pJR199 [carrying JRi]) in *L. lactis* NZ9000.

Recombinant plasmids derived from pINT29 Using primers GB-1 and GB-2, and pJP26 as the DNA template, a 507-bp fragment containing *entP* (insert JB) was obtained. The same primers were used to amplify a PCR product of 779 bp containing *entP* and *entiP* (insert JBi). The PCR fragments JB and JBi were inserted as *Bam*HI/

Table 2 Primers and PCR products used in this study

Primer or PCR product	Nucleotide sequence (5′–3′) or description[a]	Purpose
Primers		
LJ-1	CATAGAGCTCTGTAA**GGAGG**AATTTTGAAATGAG AAAAAAATTATTTAGTTTAGC	Amplification of JP and JPi
LJ-2	ATAAGTTAAGCTTGTATTAATG TCCCATACCTGCCAAACCAG	Amplification of JP
LJ-3	ATAAGTTAAGCTTGTATCAAAGTCCCGACCATGCTTTGG	Amplification of JPi
Cy5-P32uni	CGGAGGAATTTTGAAATGGC	Selection of plasmids from pMG36c
Cy5-P32rev	CCTCAACTCCAA ATATCG	As above
RL-1	GCTCATGAGAAAAAAATTATTTAG	Amplification of JR and JRi
RL-2	GCCGCCACGGCGTATTAATG TCCCATACC	Amplification of JR
RL-3	GCCGCCACGGCGTATCAAAGTCCCGACCATGC	Amplification of JRi
Nis-fw	GCTCTGATTAAATTCTG	Selection of plasmids from pNG8048e
Nis-rev	TGTTTAATTGCCATTTC	As above
GB-1	CGCGGATCCCGGTCCTCGGGATATGATAAG	Amplification of JB and JBi
GB-2	CATAGTTTAGCGGCCGCATTCTTCGTTTTCAGACTTTGCAAGC	As above
PepX_Up(*AvrBsp*)	GATCCCTAGGAGAAAGGAGGTAAATCATGAGCTTTAACCATTTTTCAATTGTTGAC	Selection of plasmids from pINT29
PepX_Down(*Spe*)	CGACTAGTTTAATTTTTCACACTTTC	As above
PCR products		
JP	261-bp *Sac*I/*Hin*dIII fragment containing the P_{32} ribosome binding site and the *entP* gene	Cloning in pMG36c
JPi	533-bp *Sac*I/*Hin*dIII fragment containing the P_{32} ribosome binding site and the *entP* and *entiP* genes	As above
JR	231-bp *Rca*I/*Bgl*I fragment containing the *entP* gene	Cloning in pNG8048e
JRi	503-bp *Rca*I/*Bgl*I fragment containing the *entP* and *entiP* genes	As above
JB	507-bp *Bam*HI/*Not*I fragment containing the *entP* gene and the upstream P_{32} promoter	Cloning in pINT29
JBi	779-bp *Bam*HI/*Not*I fragment containing the *entP* and *entiP* genes and the upstream P_{32} promoter	As above

[a]Cleavage sites for restriction enzymes are underlined in the primers; P_{32} Ribosome binding site is shown in bold

*Not*I fragments in pINT29. The ligation mixtures were used to transform *L. lactis* LL108. PCR with primers PepX_Up (*AvrBsp*) and PepX_Down (*Spe*) on selected transformants permitted identification of *L. lactis* LL108 (pJB3 [insert JB]) and *L. lactis* LL108 (pJB19 [insert JBi]). Plasmids pJB3 and pJB19 were integrated in the chromosome of *L. lactis* NZ9000 by the method of Leenhouts et al. (1998).

PCR amplification and nucleotide sequencing Oligonucleotide primers were obtained from Sigma and Biolegio. PCR-amplifications of inserts JP and JPi were performed in 50 µl reaction mixtures containing 1 µl of purified DNA, 70 pmol of each primer, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen). Samples were subjected to an initial cycle of denaturation (97°C for 2 min), followed by 35 cycles of denaturation (94°C for 45 s), annealing (50°C for 30 s), and elongation (72°C for 45 s), ending with a final extension step at 72°C for 7 min in a DNA thermal cycler Techgene (Techne). PCR-amplifications of inserts JR, JRi, JB and JBi were performed in 100 µl of reaction mixtures containing 1 µl of purified plasmid,

100 pmol of each primer, and 5 U of PWO polymerase (Roche), and included a hot start at 94°C (2 min), primer annealing at 50°C (30 s), primer elongation at 72°C (1 min), and denaturation at 94°C (45 s). Amplification reactions (35 cycles) and a final extension at 72°C for 7 min were carried out in the thermal cycler iCycler (Bio-Rad). Taq DNA polymerase (Roche) was used for colony PCR assays. The PCR-generated fragments were purified by a QIAquick PCR Purification Kit (Qiagen) or a High Pure PCR Product Purification kit (Roche) before cloning into the vectors, and for nucleotide sequencing. Nucleotide sequencing of purified PCR products was done using the ABI PRISM BigDye Terminator cycle sequence reaction kit and the automatic DNA sequencer ABI PRISM, model 377 (Applied Biosystems) at the DNA Sequencing Service Sistemas Genómicos (Valencia, Spain).

ELISA for detection and quantification of EntP Polyclonal antibodies with specificity for EntP and a noncompetitive indirect enzyme-linked immunosorbent assay (NCI-ELISA) were used to detect and quantify EntP, as previously described by Gutiérrez et al. (2004). Briefly, wells of flat-bottom polystyrene microtitre plates (Nunc) were coated overnight (4°C) with different concentrations of pure EntP or with supernatants from *E. faecium* P13 or the recombinant *L. lactis* hosts. After addition of the anti-P3-KLH serum and the goat antirabbit immunoglobulin G (IgG) peroxidase conjugate (Cappel) bound peroxidase was determined with ABTS (2,2'-azino-bis[3-ethyl-benzthiazoline-6-sulfonic acid]) (Sigma) as the substrate by measuring the absorbance of the wells at 405 nm with a Labsystems iEMS reader (Labsystems) with a built-in software package for data analysis.

Purification of EntP, and mass spectrometry analysis EntP was purified from *E. faecium* P13 and *L. lactis* IL1403 (pJP26) as previously described (Casaus et al. 1997; Gutiérrez et al. 2004). Briefly, supernatants from early stationary phase 1-l cultures were subjected to precipitation with ammonium sulfate, desalted by gel filtration, and further subjected to cation-exchange and hydrophobic-interaction chromatography, followed by reverse-phase chromatography in a fast-protein liquid chromatography system (RP-FPLC) (Amersham). Final concentrations of purified EntP were estimated using the extinction coefficient of the bacteriocin (an A_{280} of 4.3 corresponds to 1 mg ml^{-1} of EntP) and the NCI-ELISA as previously described. Purified fractions from the last reverse-phase chromatography step were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Briefly, a 1 µl sample was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 ml of a 3 mg ml^{-1} α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile was added to the dried sample, which was allowed again to air-dry at room temperature. MALDI-TOF

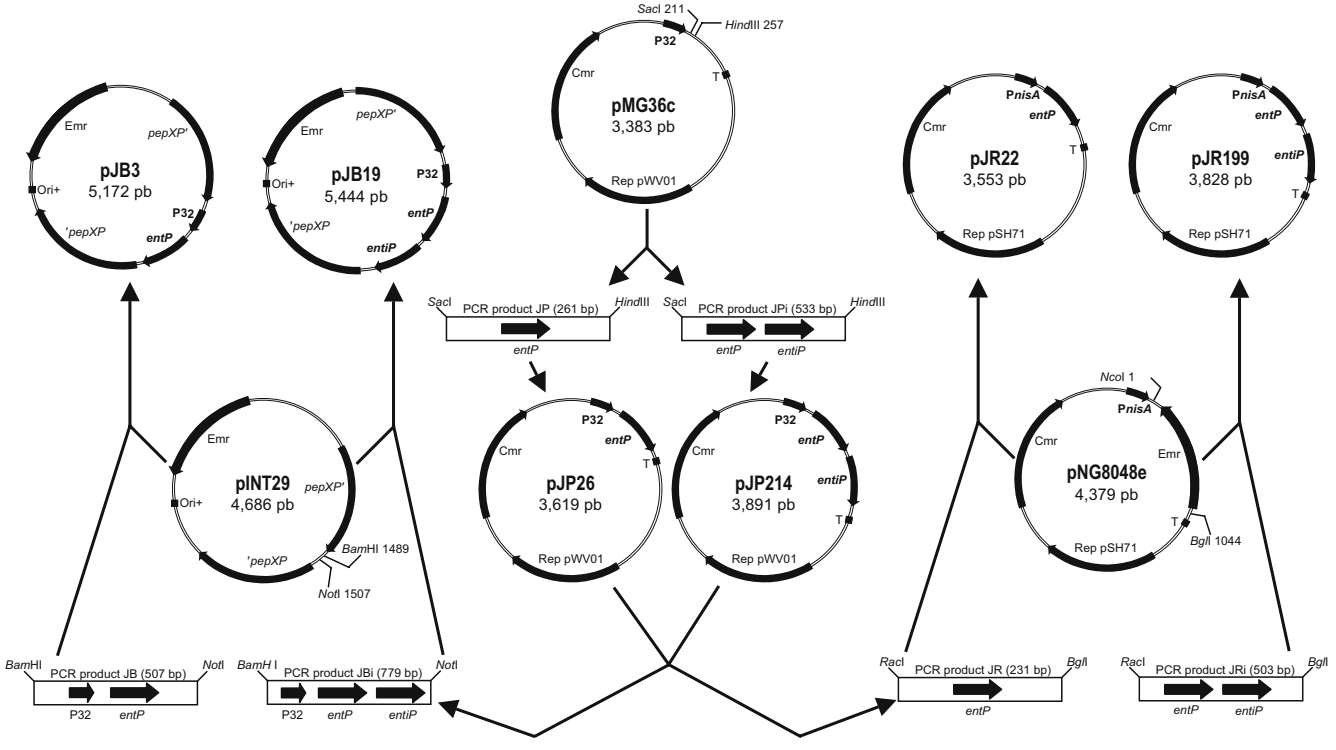

Fig. 1 Construction of the lactococcal recombinant plasmids. Sizes of plasmids are given in base pairs. Only relevant restriction enzymes are given. P_{32} constitutive lactococcal promoter, *PnisA* inducible nisin A promoter; *T* transcription terminator; *Rep* lactococcal replicons pWV01 and pSH71; Ori^+ pWV01 origin of replication; *pepXP'* and *'pepXP* 5′ and 3′ sequence of *pepX* gene, respectively; *entP* enterocin P structural gene; *entiP* enterocin P immunity gene; *Cmr* choramphenicol resistance; *Emr* erythromycin resistance

analyses were performed in a Voyager-DE STR Instrument (PerSeptive Biosystems) fitted with a nitrogen laser and operated in reflector mode, with an accelerating voltage of 25,000 V.

Protein electrophoresis, Western blotting, and overlay assay Aliquots of purified EntP, enterocin Q, and pediocin PA-1 were subjected to Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Schägger and Von Jagow (1987). Protein electrophoresis was performed on Novex 16% Tricine gels (Invitrogen) in an XCell *SureLock* Mini-Cell (Invitrogen) at 80 V constant current. Gels were stained with the Silver Stain Plus reagent (Bio-Rad) or blotted onto an Immun-Blot polyvinylidine difluoride membrane (pore size 0.2 μm) (Bio-Rad), as previously described by Gutiérrez et al. (2004). To determine the antimicrobial activity of pure bacteriocins, an overlay assay was performed, using *E. faecium* T136 as the indicator microorganism. Pure pediocin PA-1 and enterocin Q were obtained as previously described by Martínez et al. (2000) and Gutiérrez et al. (2004).

Results

Construction of the recombinant Lactococcus lactis strains for production of EntP Cloning of PCR fragments containing the pre-EntP structural gene (*entP*) with or without the EntP immunity gene (*entiP*), into the vectors pMG36c, pNG8048e, and pINT29, resulted in the plasmids pJP26 (*entP*) and pJP214 (*entP*+*entiP*), both derived from pMG36c; plasmids pJR22 (*entP*) and pJR199 (*entP*+*entiP*), both derived from pNG8048e; and plasmids pJB3 and pJB19 (*entP*+*entiP*), both derived from pINT29. The genetic map of the resulting plasmids is displayed in Fig. 1, while the various recombinant strains examined in this study are presented in Table 3.

Heterologous production and functional expression of EntP by different L. lactis strains While the supernatants of the *L. lactis* IL1403 (pMG36c), *L. lactis* NZ9000 (pNG8048e) and *L. lactis* JG1 (pINT29) control strains did not display any antagonistic effect, the supernatants of all recombinant *L. lactis* hosts showed a potent antimicrobial activity against *E. faecium* T136 (Fig. 2). Furthermore, the

Table 3 Bacteriocin production and antimicrobial activity of supernatants from recombinant *L. lactis* strains

Strain	Bacteriocin production (μg mg^{-1} cell dry weight)		Antimicrobial activity (BU mg^{-1} cell dry weight)[b]		Sp. Antimicrobial Activity (BU μg^{-1} EntP)[c]
	Enterocin P	Nisin A[a]	*E. faecium* T136	*L. lactis* MG1363	
L. lactis subsp. *lactis*					
IL1403 (pMG36c)	NP		NA		NE
IL1403 (pJP26)	12.4		921		74.3
IL1403 (pJP214)	44.6		3,572		80.1
DPC5598 (pMG36c)	NP	1.5	70	4,038	NE
DPC5598 (pJP26)	4.2	1.5	97	3,950	23.0
DPC5598 (pJP214)	5.8	1.5	115	4,125	20.0
BB24[§]	NP	2.6	116	7,020	NE
L. lactis subsp. *cremoris*					
NZ9000 (pMG36c)	NP		NA		NE
NZ9000 (pJP26)	5.6		368		65.7
NZ9000 (pJP214)	12.2		946		77.5
NZ9000 (pNG8048e)	NP		NA		NE
NZ9000 (pJR22)	23.3		7,681		330.0
NZ9000 (pJR199)	39.6		8,174		206.4
JG1	NP		NA		NE
JG5	0.7		45		68.2
JG8	1.6		119		74.4
E. faecium[d]					
P13	7.4		564		76.2
T136	NP		NA		NE

[a]Production of nisin A by *L. lactis* BB24 was calculated as previously described (Suárez et al. 1996). The production of NisA in *L. lactis* DPC5598 was estimated according to its antimicrobial activity and related to the production of NisA and the antimicrobial activity of *L. lactis* BB24

[b]Antimicrobial activity was calculated against *E. faecium* T136 (sensitive to EntP and NisA) and *L. lactis* MG1363 (sensitive to NisA and resistant to EntP)

[c]Specific antimicrobial activity refers to the antimicrobial activity against *E. faecium* T136 divided by the EntP produced

[d]Cultures of *L. lactis* BB24, *E. faecium* P13, and *E. faecium* T136 were used as controls

NP no production, *NA* no activity, *NE* not evaluable

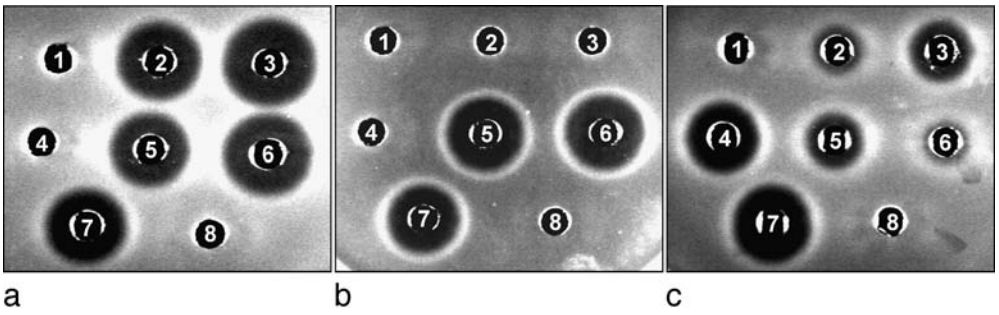

Fig. 2 Production of enterocin P by *L. Lactis* as determined by the agar well diffusion test with *E. faecium* T136 as the indicator strain. **a** Supernatants of lactococcal strains carrying constitutive promoter constructs: *1L. lactis* IL1403 (pMG36c), *2L. lactis* IL1403 (pJP26), *3L. lactis* IL1403 (pJP214), *4L. lactis* NZ9000 (pMG36c), *5L. lactis* NZ9000 (pJP26), and *6L. lactis NZ9000* (pJP214). **b** Supernatants of *L. lactis* NZ9000 carrying inducible promoter constructs: *1* and *4* pNG8048e, *2* and *5* pJR22, and *3* and *6* pJR199, before *1*, *2*, and *3* and after *4*, *5*, and *6* induction of the cultures with nisin. **c** Supernatants of *L. lactis* NZ9000 cells transformed with the integrative vectors: pINT29 (*1*), pJB3 (*2*), pJB19 (*3*); EntP from *E. faecium* P13 at 8 μg ml^{-1} (*4*), 2 μg ml^{-1} (*5*), and 0.5 μg ml^{-1} (*6*). Supernatants of *E. faecium* P13 (*7*) and *E. faecium* T136 (*8*) were used as controls

supernatants of cultures of *L. lactis* IL1403 and *L. lactis* NZ9000, carrying either pJP26 or pJP214, produced large inhibition halos, of which the ones with pJP214 were the largest (Fig. 2a). Figure 2b shows that after induction with nisin, the inhibition zones of *L. lactis* NZ9000 containing pJR22 or pJR199 are larger than those produced by *E. faecium* P13. The *L. lactis* NZ9000 derivatives *L. lactis* JG5 (pJB3) and *L. lactis* JG8 (pJB19) produce halos of inhibition which are smaller than those produced by *E. faecium* P13 (Fig. 2c). Interestingly, all *L. lactis* strains carrying vectors containing *entP* gene plus the *entiP* gene showed the largest halos of antagonistic activity.

The heterologous production of EntP by the various recombinant *L. lactis* strains was further quantified by a microtitre plate assay (MPA), and by using specific anti-EntP antibodies in an NCI-ELISA. The highest production and antagonistic activity of EntP in the supernatants of the

Table 4 Purification of enterocin P from *E. faecium* P13 and *L. lactis* IL1403 (pJP214) supernatants

Supernatant and purification stage	Volume (ml)	Total A_{254}[a]	Total activity (10^3 BU)[b]	Specific activity[c]	Increase in specific activity[d] (fold)	Yield (%)
E. faecium P13						
Culture supernatant	1,000	37,900	775	20	1	100
Fraction						
Ammonium sulfate precipitation	100	3,180	566	176	9	73
Gel filtration chromatography	200	2,200	545	248	12	70
Cation-exchange chromatography	50	21	276	13,143	657	36
Hydrophobic-interaction chromatography	10	8.95	305	34,078	1,704	40
RP-FPLC						
Fraction A	1.02	0.011	50	4,545,454	227,273	6
Fraction B	1.08	0.063	134	2,126,984	106,349	17
L. lactis IL1403 (pJP214)						
Culture supernatant	1,000	18,700	4,761	255	1	100
Fraction						
Ammonium sulfate precipitation	100	1,960	4,406	2,248	9	92
Gel filtration chromatography	200	1,160	1,603	1,382	5	34
Cation-exchange chromatography	50	10.5	292	27,809	109	6
Hydrophobic-interaction chromatography	10	9.05	330	36,464	110	7
RP-FPLC						
Fraction A	0.25	0.022	17	772,727	3,030	0.4
Fraction B	0.50	0.078	240	3,076,923	12,066	5

[a]Absorbance at 254 nm multiplied by the volume in milliliters
[b]Antimicrobial activity in bacteriocin units per milliliter (BU/ml) and multiplied by the total volume
[c]Specific activity is the number of bacteriocin units divided by the total A_{254}
[d]The specific activity of a fraction divided by the specific activity of the culture supernatant

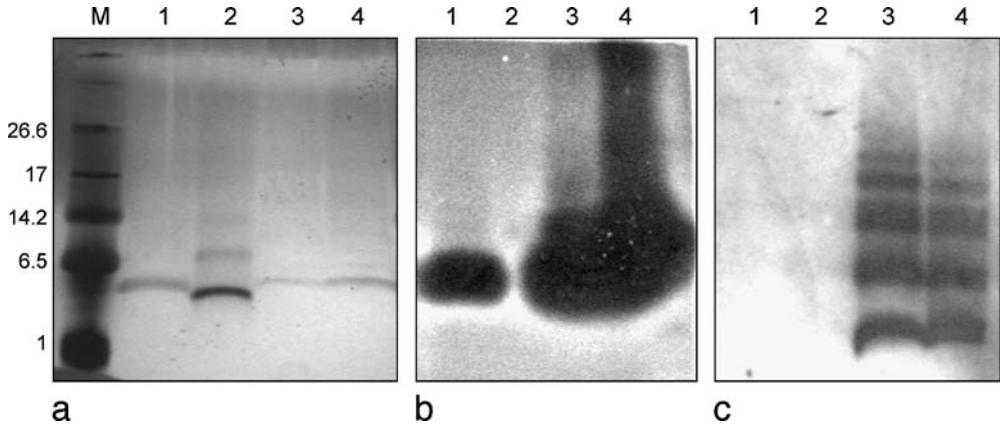

Fig. 3 Tricine-SDS-PAGE of different bacteriocins after silver staining (**a**), overlay with the indicator strain *E. faecium* T136 (**b**), and Western blotting with the anti-EntP antibodies (**c**). *M* protein molecular weight marker with sizes in kDa indicated in the *left margin*. *Lane 1* shows 1 μg of pure pediocin PA-1, *lane 2* shows 1 μg of pure enterocin Q, *lane 3* shows 1 μg of pure enterocin P from *E. faecium* P13, and *lane 4* shows 2 μg of pure enterocin P from *L. lactis* IL1403 (pJP214)

recombinant *L. lactis* cultures occurred after growth for 8 to 12 h, coinciding with the initiation of the stationary phase, after which the concentration of EntP and the activity of the supernatants decreased (results not shown). As shown in Table 3, the production of EntP in the supernatants of *L. lactis* IL1403 (pJP26) and *L. lactis* IL1403 (pJP214) was, respectively, 1.7- to 6.0-fold higher than that of the supernatant of *E. faecium* P13, while the production of EntP in the supernatants of *L. lactis* NZ9000 (pJP26) and *L. lactis* NZ9000 (pJP214) was, respectively, 0.7- to 1.6-fold higher than that of *E. faecium* P13. Interestingly, the antimicrobial activity of the supernatants of *L. lactis* IL1403 (pJP26) and *L. lactis* IL1403 (pJP214) was 2.2- to 3.6-fold higher than that in the supernatants of *L. lactis* NZ9000 (pJP26) and *L. lactis* NZ9000 (pJP214). Furthermore, the content of EntP in the supernatants of *L. lactis* NZ9000 (pJR22) and *L. lactis* NZ9000 (pJR199) was determined to be three- to five-fold higher than in *E. faecium* P13, while the antimicrobial activity was about 14-fold higher than that observed in *E. faecium* P13 supernatants. Finally, the EntP production and antimicrobial activity of the supernatants of *L. lactis* JG5 and *L. lactis* JG8 were about 10% and 20%, respectively, of those determined in the supernatant of *E. faecium* P13.

Coproduction of EntP and NisA by L. lactis DPC5598 Initial stab-on-agar tests showed that *L. lactis* DPC5598 inhibited *E. faecium* T136. The fact that it also had an antagonistic effect on *L. lactis* MG1363, which is resistant to EntP and sensitive to NisA, but not on *L. lactis* BB24, a strain resistant to EntP and NisA, suggested that *L. lactis* DPC5598 is a nisin producer. This was confirmed by PCR, in which a 184-bp fragment of *nisA* was amplified with *nisA*-specific primers from DPC5598 chromosomal DNA, and sequenced. Plasmid pJP26 or pJP214 was introduced in *L. lactis* DPC5598 and the coproduction of EntP and NisA was determined by ELISA and MPA (Table 3). The production of EntP in the supernatant of *L. lactis* DPC5598 (pJP26) and *L. lactis* DPC5598 (pJP214) was determined to be, respectively, 57% and 78% of that of *E. faecium* P13. However, the specific antimicrobial activity of the EntP produced by the *L. lactis* DPC5598 derivatives was lower than that calculated for the *L. lactis* IL1403 and *L. lactis* NZ9000 strains carrying pJP26 or pJP214. The production of NisA by *L. lactis* DPC5598 and its two $EntP^+$ derivatives was similar in all cases, but lower than that produced by *L. lactis* BB24 (Table 3).

Purification of EntP, Western blotting, and mass spectrometry analysis The results of the purification of EntP from the *E. faecium* P13 and *L. lactis* IL1403 (pJP214) supernatants are summarized in Table 4. Although the total antimicrobial activity was six-fold larger in supernatants of the latter strain, and the purification of EntP resulted in two fractions with EntP activity after RP-FPLC, the final recovery of EntP activity was 23% of the initial activity in *E. faecium* P13, and 5.4% of the initial activity in *L. lactis* IL1403 (pJP214). Further characterization of EntP in fraction B from *E. faecium* P13 and *L. lactis* IL1403 (pJP214) was performed by protein electrophoresis, Western blotting, and an overlay assay. Chemically synthesized EntQ tends to form aggregates (Fig. 3a), and pediocin PA-1 and purified EntP show strong antimicrobial activity in the overlay assay (Fig. 3b), with the largest activity displayed by EntP. After Western blotting (Fig. 3c), the antibodies with specificity for EntP only recognized reactive antigenic bands in lanes corresponding to purified EntP from *E. faecium* P13 and *L. lactis* IL1403 (pJP214). Furthermore, when the EntP fraction B purified from the two supernatants was subjected to MALDI-TOF MS analysis, a major fragment corresponding to the theoretical molecular mass of pure EntP (4,630.1) was obtained from both, *E. faecium* P13 (4,629.1) and *L. lactis* IL1403 (4,628.9) purified fractions.

Discussion

The cloning, production, and functional expression of EntP, a *sec*-dependent bacteriocin from *E. faecium* P13, has been studied in *L. lactis*. All recombinant *L. lactis* strains carrying lactococcal vectors with the EntP structural gene (*entP*) in the presence or absence of the EntP immunity gene (*entiP*) displayed extracellular antagonistic activity, suggesting that presence of *entP* is the minimum requirement for the production of biologically active EntP (Fig. 2). The production of EntP by the *L. lactis* IL1403 and *L. lactis* NZ9000 recombinant strains was generally higher than in

E. faecium P13 (Table 3). The production of EntP in *L. lactis* IL1403 hosts was higher than in *L. lactis* NZ9000 cells transformed with the same vectors. Differences in the production of bacteriocins by lactococcal strains have been previously reported (Horn et al. 1998, 1999), and may reflect unknown metabolic differences between *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* NZ9000. The specific antimicrobial activity of EntP produced by the *L. lactis* IL1403 and *L. lactis* NZ9000 derivatives was rather similar to that of EntP produced by *E. faecium* P13, suggesting that the synthesis, processing, secretion, and antagonistic activity of EntP are not affected by its heterologous production in *L. lactis*.

The coexpression of the *entP* and *entiP* genes increased the production of EntP in all *L. lactis* hosts. Increased EntP production may be explained by assuming that *L. lactis* is relatively resistant to EntP but may endure more of the bacteriocin when it expresses the EntiP product. Indeed, bacteriocin-producers are protected from their own bacteriocin by the concomitant expression of a cognate immunity protein. These proteins are located intracellularly and/or in the cytoplasm, and act either by affecting bacteriocin aggregation and pore formation, or by disturbing the interaction between the bacteriocin and a membrane-located bacteriocin receptor (Venema et al. 1994). In a comparative study of immunity proteins, the functionality of the EntiP of *E. faecium* P13 as an immunity protein was confirmed (Fimland et al. 2002). Alternatively, it may be that EntiP participates in the processing, secretion and/or transport of EntP in *L. lactis*.

The level of production of EntP also depended on the lactococcal vector used (Table 3). The enhanced EntP production in cells with the nisin-inducible constructs may be due to copy number differences between pNG8048 and pMG36c (Kok et al. 1984; De Vos 1987), but, more likely, is caused by the different promoters used to drive gene expression. Interestingly, the specific antimicrobial activity of EntP produced by *L. lactis* NZ9000 (pJR22) and *L. lactis* NZ9000 (pJR199) is higher than that observed in any other *L. lactis* host or in *E. faecium* P13, confirming the potential of the nisin-controlled expression system for the overproduction and stability of (heterologous) proteins, or, as in this case, small (antimicrobial) peptides (De Ruyter et al. 1996). The increased specific antimicrobial activity observed for EntP may be ascribed to the short induction time for EntP production (3 h), which, most probably, prevented secreted EntP from attaching to cell walls to form aggregates and/or to undergo protease-degradation. The ten-fold-lower yields of EntP in the supernatants of *L. lactis* JG5 (pJB3) and *L. lactis* JG8 (pJB19) are in agreement with the fact that pINT129 (a derivative of pINT29) leads to single copy integrations in *L. lactis* MG1363 (Leenhouts et al. 1998). Although *L. lactis* JG5 and *L. lactis* JG8 produce lower amounts of EntP, they may provide a more stable EntP production platform, without the need for selective antibiotic pressure, which may be considered safer for the production of EntP as a natural preservative or ingredient for the food industry.

L. lactis subsp. *lactis* DPC5598 was selected as a host for production of EntP because it is an easily transformable, plasmid-free derivative of an industrial strain that is extensively used because of its phage insensitivity and fast-acid producing ability (Trotter et al. 2002). We show here that it produces NisA; and thus, the coproduction of NisA and EntP was further investigated (Table 3). The lower production of EntP by *L. lactis* DPC5598 (pJP26) and *L. lactis* DPC5598 (pJP214) compared to that of *L. lactis* IL1403 and *L. lactis* NZ9000 carrying the same plasmid may be attributed to the production of more than one bacteriocin in multibacteriocinogenic hosts (Gutiérrez et al. 2004). The lower specific antimicrobial activity of EntP produced by the *L. lactis* DPC5598 recombinant strains may be associated to genomic differences of this strain with *L. lactis* IL1403, and related to a higher extracellular proteinase activity of the former. The lower production of NisA by recombinant *L. lactis* DPC5598 strains as compared to that of *L. lactis* BB24 may be ascribed to unknown genetic and/or metabolic differences between the strains. In any case, the *L. lactis* DPC5598 derivatives generated in this study were able to express and secrete NisA together with EntP. While the use of pure bacteriocins is currently a controversial issue, employing "food-grade" organisms as producing strains may provide a means by which the potential benefits of these antimicrobial compounds can be exploited (Horn et al. 1999).

During purification of EntP from the supernatants of *L. lactis* IL1403 (pJP214) and *E. faecium* P13, two distinct chromatographic fractions were obtained (Table 4). The existence of multiple chromatographic peaks after purification of EntP and other bacteriocins, such as nisin Z, pediocin PA-1, and carnobacteriocin BM1, has been ascribed to the coexistence after RP-FPLC of oxidized and nonoxidized forms of the same bacteriocin (Gutiérrez et al. 2004). They may also occur due to the formation of EntP aggregates or aggregates of EntP to media components. Further work should be performed to analyze conditions that cause the separation of EntP into two fractions. However, the recovery of EntP from the *L. lactis* (pJP214) supernatant was lower than that from *E. faecium* P13. If the food industry would demand large quantities of EntP, overproduction by heterologous hosts would not be useful without the development and optimization of more efficient purification procedures for this bacteriocin. EntP purified from either *E. faecium* P13 or *L. lactis* IL1403 (pJP214) has a strong tendency to form high molecular mass aggregates with antimicrobial activity (Fig. 3). Similarly, purified EntP from both cultures gives, after MALDI-TOF MS analysis, a major fragment with a molecular mass corresponding to the theoretical molecular mass of EntP. Whereas production of EntP by recombinant *E. coli* cells was rather low as compared to that of *E. faecium* P13 (Gutiérrez et al. 2005) the results presented here show that the *E. faecium* signals driving the processing and secretion of EntP work adequately and efficiently in *L. lactis*.

Two other bacteriocins, acidocin B produced by *Lactobacillus acidophilus* M46 (Van der Vossen et al. 1994) and

divergicin A produced by *Carnobacterium divergens* LV13 (Worobo et al. 1995), have been heterologously produced by LAB using the general secretory pathway, but a precise quantitation of their production was not performed. In-frame fusions of the *sec*-dependent signal peptide of divergicin A with the mature sequence of carnobacteriocin B2 (McCormick et al. 1996), brochocin C (McCormick et al. 1998), colicin V (McCormick et al. 1999), and enterocin B (Franz et al. 1999b) have also permitted production of these bacteriocins in the supernatants of different LAB hosts, including *L. lactis*. It remains unclear why most LAB bacteriocins have a dedicated secretion and processing system when they can access the general secretory pathway if provided with an appropriate signal peptide.

The production of EntP in the supernatants of most of the recombinant *L. lactis* strains and, above all, in those of the high-level producers *L. lactis* subsp. *lactis* IL1403 (pJP214) and *L. lactis* subsp. *cremoris* NZ9000 (pJR199) is, in most of the cases, comparatively higher than production of a number of other bacteriocins in different LAB hosts using dedicated ABC transport systems (Chikindas et al. 1995; Van Belkum et al. 1997; Axelsson et al. 1998; Biet et al. 1998; Horn et al. 1998, 1999; Martínez et al. 2000; Kawamoto et al. 2002; Morisset and Frere 2002) or the *sec* pathway (Worobo et al. 1995; Biet et al. 1998; McCormick et al. 1998, 1999), indicating that production of EntP by the *sec*-dependent pathway is an efficient process in *L. lactis*. Protein secretion is a preferred means of protein expression in the development of LAB as vehicles for the delivery of biologically active molecules (Dieye et al. 2003). The high-level production of active EntP in the supernatants of *L. lactis* suggests that fusions between the signal peptide of EntP and the mature parts of other bacteriocins may allow *L. lactis* to secrete bacteriocins in the absence of specific immunity and secretion proteins. The signal peptide of EntP may also serve as a model for secretion by *L. lactis* and other LAB of numerous peptides or proteins of interest to the food industry.

Acknowledgements The authors wish to express their gratitude to Prof. R. P. Ross (Teagasc Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) for providing the strain *L. lactis* DPC5598. This work was partially supported by grants 07G/0026/2000 and S-0505/AGR/0265 from the Comunidad de Madrid, and AGL2000-0707 and AGL2003-01508 from the Ministerio de Educación y Cultura, Spain. J. Gutiérrez is the recipient of a fellowship from the Ministerio de Ciencia y Tecnología (MCYT), Spain.

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